Corticotropin-releasing factor receptor 1 mediates acute and delayed stress-induced visceral hyperalgesia in maternally separated Long-Evans rats

Ines Schwetz,1,4 James A. McRoberts,1,4 Santosh V. Coutinho,1,4 Sylvie Bradesi,1,4 Greg Gale,3 Michael Fanselow,3 Mulugeta Million,1,4 Gordon Ohning,1,4 Yvette Taché,1,4 Paul M. Plotsky,5 and Emeran A. Mayer1,2,4

1Corticotropin Releasing Factor Research Program, Center for Neurovisceral Sciences and Women’s Health, and Digestive Diseases Research Center, Department of Medicine, 2Center for Ulcer Research and Education/Department of Physiology, Psychiatry and Biobehavioral Sciences, and 3Center for Ulcer Research and Education/Department of Psychology, David Geffen School of Medicine, University of California-Los Angeles, and 4Greater Los Angeles Veterans Affairs Healthcare System, Los Angeles, California; and 5Stress Neurobiology Laboratory, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia

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Schwetz, Ines, James A. McRoberts, Santosh V. Coutinho, Sylvie Bradesi, Greg Gale, Michael Fanselow, Mulugeta Million, Gordon Ohning, Yvette Taché, Paul M. Plotsky, and Emeran A. Mayer. Corticotropin-releasing factor receptor 1 mediates acute and delayed stress-induced visceral hyperalgesia in maternally separated Long-Evans rats. Am J Physiol Gastrointest Liver Physiol 289: G704–G712, 2005. First published June 30, 2005; doi:10.1152/ajpgi.00498.2004.—In rodents, maternal pup interactions play an important role in programming the stress responsiveness of the adult organism. The aims of this study were 1) to determine the effect of different neonatal rearing conditions on acute and delayed stress-induced visceral sensitivity as well as on other measures of stress sensitivity of the adult animal; and 2) to determine the role of corticotropin-releasing factor receptor (CRF-R) subtype 1 (CRF1R) in mediating visceral hypersensitivity. Three groups of male Long-Evans rat pups were used: separation from their dam for 180 min daily from postnatal days 2–14 (MS180), daily separation (handling) for 15 min (H), or no handling. The visceromotor responses (VMR) to colorectal distension, stress-induced colonic motility, and anxiety-like behavior were assessed in the adult rats. The VMR was assessed at baseline, immediately after a 1-h water avoidance (WA) stress, and 24 h poststress. Astressin B, a nonselective CRF-R antagonist, or CP-154,526, a selective CRF1R antagonist, was administered before the stressor and/or before the 24-h measurement. MS rats developed acute and delayed stress-induced visceral hyperalgesia. In contrast, H rats showed hypoalgesia immediately after WA and no change in VMR on day 2. MS rats with visceral hyperalgesia also exhibited enhanced stress-induced colonic motility and increased anxiety-like behavior. In MS rats, both CRF-R antagonists abolished acute and delayed increases in VMR. Rearing conditions have a significant effect on adult stress responsiveness including immediate and delayed visceral pain responses to an acute stressor. Both acute and delayed stress-induced visceral hypersensitivity in MS rats are mediated by the CRF/CRF1R system.

Address for reprint requests and other correspondence: E. A. Mayer, Center for Neurovisceral Sciences and Women’s Health, VAGLAHS, Bldg. 115, Rm. 223, 11301 Wilshire Blvd., Los Angeles, CA 90073 (e-mail: emayer@ucla.edu).

IRRITABLE BOWEL SYNDROME (IBS) is a common gastrointestinal disorder characterized by abdominal pain or discomfort associated with changes in bowel frequency or consistency (58). Altered gut motility, enhanced perception of visceral stimuli (“visceral hypersensitivity”), and stress sensitivity are thought to be important factors in the pathophysiology of IBS (11). Numerous reports in the literature provide evidence for the prominent involvement of stress in the pathophysiology (53) and clinical presentation of IBS (32). For example, early life stressors associated with neglect, loss of primary caregiver, and physical or sexual abuse have been shown to increase the risk of developing IBS later in life (27). Furthermore, early aversive life events are known to lead to long-lasting stress hyperresponsiveness, presumably due to corticotropin-releasing factor (CRF) hypersecretion (16, 17).

Maternal separation is a well-characterized model of early life stress in rodents, and a variety of different procedures and separation schedules have been used by different laboratories (25). The maternal separation model is built on the concept that interference with normal dam-litter interactions can produce profound and long-lasting changes in the development of the central nervous system (CNS), including systems that regulate stress responsiveness (22). One problem with reported maternal separation models, in particular in their comparability and reproducibility (43, 44), is the variations in subtle and potentially crucial details of the procedures: the selected comparison group, rat strain, and a range of other poorly characterized factors such as maternal competency of the dams, prenatal stress history, and quality of animal facilities. In the current study, we wanted to assess the variability of the expected phenotype by studying a large number of these animals from different generations.

In the maternal separation paradigm originally reported by Plotsky and Meany (42) and used in the current study, daily separation of the entire litter of Long-Evans rat pups from the dam for 180 min gives rise to a phenotype in the offspring characterized by a long-lasting dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis reactivity to stress (21) comparable with findings in human patients that experienced adverse early life events (17). Neurochemical changes in adult animals include increased release of CRF in response to stress and altered expression of glucocorticoid receptors as well as changes in the norepinephrine and GABA systems (2, 5, 18, 22, 42). Adult offspring are also known to exhibit behavioral alterations such as enhanced anxiety-like behaviors (19, 21),

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anhedonia (31), increased ethanol preference (19), and an increased risk to develop depression-like behaviors (19, 22). In a different procedure, known as handling, the experience of short separations from the dam (usually 15 min) during neonatal life results in a decreased responsiveness to stress (20, 22). This change is reflected by decreased HPA reactivity (34), increased GABA<sub>A</sub>/central benzodiazepine receptor binding in several anatomic areas in the brain (5), reduced activity of the brain stem noradrenergic system (26), and alterations in glucocorticoid receptor expression in the hippocampus (23).

Several recent reports have noted changes in gastrointestinal function in adult rats previously exposed to neonatal stress. For example, Soderholm et al. (51) have provided evidence for alterations in colonic permeability to macromolecules and bacterial antigens in maternally separated rats. Maternal deprivation was also associated with an enhanced inflammatory response following chemical induction of experimental colitis with trinitrobenzene sulfonic acid (4). We have previously reported that early life stress in the form of a 180-min maternal separation (MS180) predisposes adult Long-Evans rats to develop acute stress-induced visceral hypersensitivity (SIVH) and increased colonic motility in response to a psychological stressor (9).

CRF plays a primary role in coordinating the body’s overall response to stress (3, 33). Intracerebroventricular administration of CRF produces behavioral, physiological, and immunological responses similar to those induced by stress (40, 56). Inhibition of CRF-mediated responses by antagonists (53), or by genetic means in knockout animals (50), result in a decrease in the animals’ response to stress. Several reports implicate the CRF/CRF receptor subtype 1 (CRF,R) system in the mediation of visceral hyperalgesia in response to acute physical and psychological stressors. For example, the central administration of CRF increases the visceromotor response (VMR) to colorectal distension (CRD) in rats (15). We have recently demonstrated that the CRF,R antagonist CP-154,526 prevents the development of delayed visceral hyperalgesia 24 h after acute water avoidance (WA) stress in adult Wistar rats (49). Furthermore, NBI-35965, a selective CRF,R antagonist, abolished acute SIVH in male Long-Evans rats that had undergone maternal deprivation (37).

In the current report, we wanted to further characterize the effect of neonatal rearing conditions [MS180; handled (H); and nonhandled (NH)] on adult stress modulation of nociceptive responses to visceral stimuli by studying a large population of animals obtained from different generations. Specifically, we wanted to address the following questions: 1) What is the effect of these rearing conditions (MS180, H, and NH) on visceral sensitivity in the adult rat following an acute stressor and 24 h later? 2) What percentage of animals in the MS180 group develops alterations in visceral nociceptive responses? 3) Does the subset of MS180 rats developing SIVH also show other evidence for enhanced stress responsiveness? and 4) Are acute and delayed SIVH in MS180 rats dependent on the CRF/CRF,R system?

**METHODS**

**Animals**

All procedures were carried out in accordance with the United States Public Health Service Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Greater Los Angeles Veterans Affairs Healthcare System. We used a procedure for mother-infant separation in Long-Evans rats that has been shown to result in SIVH (9). For this study, primiparous dams were ordered at three different time points in time, and experiments were replicated at three different time points in time. On postnatal day (PND) 2, pups were sexed and female pups were culled. Standardized litters of seven to eight pups were randomly assembled and assigned to foster dams and to one of three rearing conditions. This procedure, which could result in one or more of the pups ending up with their own mom, was followed equally for dams in all three conditions. A total of nine litters were subjected to MS180, three litters to NH, and another three litters to H treatment, with each litter being made up of seven to eight pups. For 180-min maternal separation (MS180), dams were removed from the maternity cages and each placed in separate cages for 180 min daily from PND 2–14 inclusive. The litters were then removed as a group from their home cage and placed (as an intact litter) in isolation cages in a neonatal incubator set to 32 ± 0.5°C (9). At the end of the separation period, the litter was returned to the maternity cages before returning the dam. The H group experienced a similar procedure, however, the pups remained away from the dam for only 15 min daily (PND 2–14). The NH group was left undisturbed until weaning. Routine cage cleaning was suspended for all groups from delivery until PND 14. Approximately 50% of the soiled bedding material in all maternity cages was replaced with clean bedding and mixed well no more than once per week as described previously (9). On PND 21, pups were weaned (Fig. 1, time line) and litter housed until PND 30, at which time they were housed in same-treatment pairs. All animals were maintained on a 12:12-h light-dark cycle (lights on at 6 AM) and had free access to food and water.

**Response to Novelty: Open Field**

At 6 wk of age, rats were exposed to an open field (90 × 60 × 45 cm) located in a dimly lit room. For 4 min, rats moved around in relative darkness. After 4 min, three light bulbs (100 W) were switched on. The light period also lasted for 4 min. The whole session was videotaped by a camera suspended from the ceiling. The number of crossings on a grid on the floor of the cage during the light and the dark phases were counted after the session. The experimenter was blinded to the rearing condition of the rats.

**Assessment of Colonic Motility After Stress**

At 8 wk of age, all rats were placed in novel Plexiglas cylinders in which they were awake and loosely restrained. Fecal pellets were counted after 10 min.
Assessment of Visceral Sensitivity

Surgery. As previously described (8), adult rats were deeply anesthetized with pentobarbital sodium (45 mg/kg, Nembutal) administered intraperitoneally. Electrodes (teflon-coated stainless steel, Astrazeneca, Sweden) for electromyographic (EMG) recording were stitched into the external oblique musculature, just superior to the inguinal ligament. The fistula housing the electrode leads was externalized through a 4-mm incision on the left side of the abdominal wall for future access. After surgery, rats were housed in pairs and allowed to recuperate for at least 7 days. Wounds were tested for tenderness to ensure complete recovery before rats were tested.

Colorectal distension. The visceral stimulus employed was distension of the descending colon and rectum using a method that has been previously described (39). Briefly, animals were lightly anesthetized with halothane and a flexible latex balloon (6 cm) lubricated with Surgilube (E. Fougera, Melville, NY) was inserted intra-anally into the rectum and descending colon. The balloon was positioned such that its end was located 1 cm proximal to the anus and was secured in place by taping the balloon catheter to the base of the tail. Animals were allowed to recover from anesthesia for ~30 min. The balloon pressure was continuously monitored online with the aid of a customized pressure control device (AstraZeneca R&D, Moehlndal, Sweden). CRD in awake rats results in contraction of the abdominal and hindlimb musculature, i.e., a VMR (39), which is recorded as EMG activity in the external oblique musculature. Each distension lasted 20 s at 4-min interstimulus intervals. EMG activity was recorded 20 s before CRD, 20 s during CRD, and 20 s after termination of CRD. The EMG activity was rectified, and the area under the curve (AUC) of the integrated EMG response was analyzed.

Baseline responses to graded intensities of phasic CRD (10, 20, 40, 60, 80 mmHg) were obtained. The animals were then exposed to 1-h WA stress (see WA stress). Responses to phasic CRD were obtained immediately after WA and again 24 h later.

WA stress. Plastic tanks (25 × 25 × 45 cm) were filled with fresh tap water at room temperature (22°C) to within 1 cm of the top of a pedestal (8 × 8 × 10 cm) that is attached to the bottom of the tank. Rats were placed on the pedestal and left undisturbed for a period of 1 h. This well-characterized test represents a psychological stressor that leads to large elevations of ACTH and corticosterone within 30 min (38).

CRF-R antagonist experiments. In a separate set of experiments, MS180 rats with robust increases in VMR to CRD after stress were selected (ratio of AUC of poststress VMR/AUC of baseline VMR > 1.25) for further testing. The experimental design was the same as described above (baseline CRD; 1-h WA stress, poststress CRD, CRD 24 h after WA on day 2).

The first group (n = 6) was injected with astressin B (20 µg/kg), a nonselective CRF-R antagonist that does not cross the blood-brain barrier, intracisternally 15 min before the start of WA stress, and again 15 min before the start of the 24 h CRD. The vehicle group (n = 8) was injected with saline intracisternally at the same time points.

CP-154,526 (32 mg/kg), a selective CRF1R antagonist that freely crosses the blood-brain barrier, was injected subcutaneously 45 min before WA (group 3, n = 11). Group 4 (n = 17) was treated with vehicle. Group 5 was injected with CP-154,526 (32 mg/kg sc) 1 h before the start of CRD on day 2 (n = 8). The last group of MS180 rats was treated with vehicle.

Drugs. CP-154,526 was kindly provided to us by Pfizer. It was dissolved in 5% DMSO, 5% Cremophor EL, and 90% saline as described previously (35). Astressin B (Clayton Foundation Laboratories, Salk Institute, La Jolla, CA) was dissolved in pyrogen-free distilled water.

Data analysis and statistics. One-way analysis of variance with Tukey’s posttest for multiple comparisons was used to compare open-field and fecal pellet output after stress. The AUC of the raw EMG amplitude response as a function of pressure was calculated for each animal for each condition (baseline, post-WA and 24 h). Ratios were calculated for each rat by dividing the AUC of the poststress measurement by the AUC of baseline. The ratios were compared with a hypothetical value of 1 using Wilcoxon’s sign-rank test. One-way analysis of variance with Tukey’s posttest for multiple comparisons was used to compare the three groups. To compare two conditions (drug vs. vehicle), an unpaired t-test was used. Throughout the study, P < 0.05 was considered significant.

RESULTS

Effect of Rearing Conditions on Stress-Induced Modulation of Visceral Responses to Noxious Visceral Stimuli

To determine the effect of rearing conditions on stress-induced modulation of visceral nociception in adult animals, CRD was performed at baseline, immediately following 1 h of WA stress, and 24 h after the acute stressor (day 2). In MS180 rats (n = 55 pups from 9 litters), WA resulted in an acute 16% increase in VMR (1.16 ± 0.05). In contrast, H rats (n = 24 pups from 3 litters) showed a significant 20% decrease in VMR immediately after WA (0.80 ± 0.06, P < 0.05). There was no statistically significant change in VMR in NH rats (n = 18 pups from 3 litters; 0.90 ± 0.08) immediately after WA. The difference in VMR immediately after WA between MS180 and both NH and H rats was statistically significant (P < 0.05, Fig. 2). On day 2, both MS180 and NH rats showed a robust increase in VMR (1.53 ± 0.08 and 1.35 ± 0.15, respectively). In contrast, there was no significant change in H rats (1.07 ± 0.10). The difference in VMR between MS180 and H rats was significant (P < 0.05).

Interindividual Differences in Stress-Induced Modulation of VMR

To determine interindividual differences in SIVH among the three rearing groups, we determined the percentage of animals
in each group that showed either hyperalgesia or hypoalgesia in response to the acute WA stress. Sixty-four percent (35 of 55) of MS180 rats exhibited an increase in VMR (ratio of H11022/1) immediately after WA, consistent with acute SIVH (Table 1). In contrast, 83% (20 of 24) of H rats developed a decrease in VMR (ratio of H11021/1) immediately after WA, consistent with stress-induced visceral hypoalgesia. Seventy-six percent (42 of 55) of MS180 rats and 55% (7 of 18) of NH rats developed an increase in VMR on day 2, consistent with delayed SIVH.

### Table 1. Effect size of acute and delayed SIVH in MS180, NH, and H rats

<table>
<thead>
<tr>
<th>Ratio (AUC of poststress VMR/AUC of baseline VMR)</th>
<th>≤1.0</th>
<th>1.01–1.5</th>
<th>1.51–2.0</th>
<th>≥2.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS180, % Post-WA</td>
<td>36</td>
<td>46</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Day 2</td>
<td>24</td>
<td>34</td>
<td>24</td>
<td>18</td>
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<tr>
<td>NH, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-WA</td>
<td>61</td>
<td>28</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>45</td>
<td>22</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>H, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-WA</td>
<td>83</td>
<td>13</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>54</td>
<td>25</td>
<td>21</td>
<td>0</td>
</tr>
</tbody>
</table>

The overall effect of water avoidance (WA) was determined by calculating the area under the curve (AUC) for the stimulus-response functions obtained at baseline, immediately after WA, and on day 2. Data are percentages of MS180, handled (H), and nonhandled (NH) rats. SIVH, stress-induced hypersensitivity; VMR, visceromotor response.

### Stress-Induced Fecal Pellet Output/Colonic Motility Changes

Stress-induced increases in colonic motility, mediated by central CRF1R, have been reported in rat models of stress (30, 55). To determine whether differences in stress responsiveness in the adult rat are also reflected in differences in stress-induced colonic motility, we evaluated the effect of 10 min restraint stress on fecal pellet output in MS180 (n = 70 pups from 9 litters), NH (n = 23 pups from 3 litters), and H (n = 38 pups from 3 litters) rats. Post hoc comparisons indicated that restraint stress increased fecal pellet output 35% in MS180 compared with H rats (4.2 ± 0.2 vs. 3.1 ± 0.3, P < 0.05; Fig. 3). In contrast, there was no significant difference between MS180 and NH rats (3.8 ± 0.4).

### Anxiety-Like Behavior

At 6 wk of age, rats were exposed to the open-field test (1). The number of grid-line crossings in the dark and the light periods was recorded.

In the dark phase, NH rats (n = 24 rats from 3 litters) exhibited significantly fewer crossings compared with either MS180 rats (51.3 ± 2.0 vs. 59.5 ± 1.6, n = 63 rats from 9 litters, P < 0.05) or H rats (65.1 ± 1.6, n = 38 rats from 3 litters, P < 0.001). Similarly, in the light phase, NH rats moved significantly less compared with either MS180 rats (16.7 ± 1.8 vs. 28.5 ± 1.5, P < 0.001) or H rats (34.3 ± 2.2, P < 0.001; Fig. 4A). Because anxiety is typically associated with de-

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Fig. 3. Stress-induced colonic motility. Fecal pellet output after 10 min of restraint stress in MS180 (n = 71), NH (n = 23), and H (n = 38) rats is shown. MS180 rats expelled significantly more fecal pellets than H rats in response to restraint stress. *P < 0.05 compared with NH rats.

Fig. 4. Anxiety-like behavior. Open-field tests in MS180 (n = 63), NH (n = 24), and H (n = 38) rats are shown. A: crossovers. NH rats moved significantly less in both the light and dark phases compared with both MS180 and H rats. *P < 0.01 compared with H and MS180 rats. B: thigmotaxis. There was a significant increase in %wall behavior for all 3 groups in the light phase (P < 0.0001). Each of the 3 handling conditions differed reliably from each other (P values <0.0003), with NH rats showing more thigmotaxis compared with both MS180 and H rats.
increased movement in the open field, the MS180 rats at this age did not respond with a profile consistent with a general increase in anxiety on this particular test.

To confirm these results, we analyzed thigmotactic behavior of the animals during the open-field test. Thigmotaxis, the tendency to stay near walls and other objects, increases when rodents are anxious or fearful (14). Therefore, we analyzed the time spent near the wall by dividing the chamber into two sections of approximately equal area, an 8.5-cm perimeter and the remaining center rectangle. As expected for an increase in anxiety, the onset of light caused an increase in thigmotaxis (percent wall behavior) for all groups, $F(1,123) = 103.74, P < 0.0001$. There was also a reliable difference for the different rearing conditions, $F(2,123) = 25.46, P < 0.0001$ (see Fig. 4B). Each of the three handling conditions differed reliably from each other ($P$ values <0.0003). Thigmotaxis in the MS180 rats was intermediate between the H and NH rats, which showed the highest levels of thigmotaxis. Therefore, thigmotaxis and crossovers are consistent in that anxiety is highest in the NH animals, as opposed to the MS180 rats.

**Differences in Colonic Motility and Anxiety-Like Behavior in MS180 Rats With and Without Visceral Hyperalgesia**

To assess whether MS180 rats, with or without SIVH, also differ on other measures of enhanced stress responsiveness, we compared colonic motility and anxiety measures between the two groups. MS180 rats were divided into two groups: those with post-WA and 24-h ratios above one (“hyperresponders”) and the remaining animals (“hyporesponders”). MS180 rats that exhibited visceral hypersensitivity ($n = 20$ rats from 9 litters) expelled a significantly higher number of fecal pellets after restraint stress than hyporesponders ($n = 14$ rats from 8 litters, $5.5 \pm 0.4$ vs. $4.1 \pm 0.4, P < 0.05$). Hyperresponsive MS180 rats ($n = 14$) moved less in the light phase (i.e., exhibited fewer crossings) than hyporesponsive rats ($n = 18$, $26.36 \pm 1.95$ vs. $33.39 \pm 2.2, P < 0.05$). There was no difference between the two groups for the number of crossings in the dark phase. Grouping the MS180 rats in to hyper- and hyporesponders with respect to SIVH represents post hoc analysis of the data rather than an a priori testing of a hypothesis. Thus the results need to be viewed with caution.

**Effect of CRF-R Antagonists on SIVH in MS180 Rats**

The effect of the nonselective CRF-R antagonist astressin B on SIVH was assessed by injecting the compound before WA and before CRD on day 2. Astressin B significantly decreased acute SIVH compared with vehicle (0.69 ± 0.11 vs. 1.39 ± 0.17, $P < 0.05$). Similarly, delayed SIVH was abolished by astressin B compared with vehicle (0.83 ± 0.1 vs. 1.86 ± 0.4, $P < 0.05$), as illustrated in Fig. 5A.

The selective CRF$_1$R antagonist CP-154,526 (32 mg/kg injected before WA) significantly decreased acute SIVH in MS180 rats (0.94 ± 0.09 vs. 1.45 ± 0.14, $P < 0.05$) compared with vehicle. A single injection of CP-154,526 (32 mg/kg before WA) tended to decrease delayed SIVH compared with vehicle. However, as shown in Fig. 5B, this decrease failed to reach significance ($1.1 \pm 0.18$ vs. $2.2 \pm 0.46, P = 0.09$).

Administration of CP-154,526 1 h before the start of CRD on day 2 led to a significant decrease in delayed SIVH compared with vehicle (0.93 ± 0.09 vs. 1.22 ± 0.03, $P < 0.05$).

**DISCUSSION**

By studying a large population of adult Long-Evans rats with a history of different rearing conditions, we demonstrated that daily separation of the intact litter from their moms for 3 h during PND 2–12 favors a phenotype with a greater degree of SIVH. In contrast, daily handling associated with separation for 15 min gives rise to a phenotype that exhibits an acute decrease in VMR to CRD, consistent with stress-induced visceral hypalgesia. MS180 rats with SIVH also showed increased colonic motility responses to stress and an increase in anxiety-like behavior, consistent with a generalized increase in stress responsiveness. SIVH in MS180 rats was sensitive to central administration of a nonselective CRF-R antagonist and...
to peripheral administration of a CNS-penetrable CRF1R selective antagonist, suggesting a role for the upregulation of CRF/CRF1R signaling in mediating SIVH in this animal model.

What Is the Effect of Rearing Conditions on Viscerosensitivity in the Adult Rat Following an Acute Stressor?

Our findings of SIVH in a large number of rats from a total of nine litters reared under the MS180 conditions confirm our previous findings obtained in a much smaller sample (9). In addition, we now demonstrate that the MS180 rats not only differ in their CRD-induced response to an acute stressor but also show even greater visceral hyperalgesia when assessed 24 h after the stressor. Although both NH and MS180 rats showed the delayed SIVH, both the percentage of animals demonstrating this response and its overall magnitude were significantly greater in the MS180 animals. Approximately 60% of the MS180-reared animals showed SIVH following the acute stressor, whereas this number increased to 75% 24 h later. The corresponding numbers in NH rats were 39% and 55%. In contrast, handling associated with brief daily separations led to a decrease in VMR immediately after stress without any change when assessed 24 h later. Therefore, the effect of the 3-h daily maternal separation intervention is best viewed as a factor in shifting the normal distribution of stress responsiveness toward the hyperresponsive side of the spectrum, whereas brief daily handling seems to lead to a shift toward stress hyposensitivity. This is consistent with reports in the literature demonstrating that the handling procedure is associated with a reduced stress responsiveness, as manifested by a decreased HPA axis reactivity (34), an increased GABA_A/central benzodiazepine receptor binding in the brain, and increased exploration in a novel open field (5).

The variability of SIVH among the animals was seen both within and between litters of a given generation as well as between litters from different generations. Because we did not record maternal behaviors in our studies, one can only speculate that differences in the quality of care that dams provided for individual pups and differences between individual dam behaviors contributed to our findings (12). In the current study, nine litters were used in the maternal separation paradigm and experimental groups were always made up of rats from different litters. If experimental groups were set up with a disproportional number from a particular dam, the results may be biased by the unique behavior of that particular dam. Such a litter-related bias would be particularly problematic if a small number of litters is used and if the results are erroneously assigned to the maternal separation effects. Another potentially confounding factor is the recently reported finding that increased maternal care is induced when moms receive an entire litter of foster pups (10). In the current study, the litters were assembled in such a way that they were primarily made up of foster pups, although a mom may have ended up with one or more pups of her own litter. Because this technique was equally used for all three rearing conditions, this variable in maternal behavior is unlikely to have influenced the observed group differences.

Is the Effect of Rearing Conditions on Visceral Sensitivity Related to a Generalized Effect on Stress Responsiveness?

The effect of rearing conditions on visceral sensitivity may be specific to the gut (e.g., neurogenic gut inflammation) or it may be part of a more generalized alteration in CNS responsiveness. If the latter hypothesis is correct, one would expect that 1) the MS180 group (compared with the NH and H groups) and 2) the hypersensitive animals within the MS180 group would show other features of altered CNS responsiveness such as altered stress-induced fecal pellet output and anxiety-like behavior. The current findings support this hypothesis: stress-induced fecal pellet output, a well-characterized measure of stress-induced colonic motility increased by sacral parasympathetic pathways (54), was greater in the MS180 group compared with the other two rearing conditions and greater in the MS180 group with visceral hypersensitivity compared with the group without SIVH.

These findings are consistent with a series of studies demonstrating enhanced autonomic nervous system and HPA axis responsiveness in MS180 rats [summarized in Ladd et al. (22) and Milde et al. (36)] as well as with elegant studies demonstrating the relationship between specific maternal behaviors in dam-pup interactions in the development of glucocorticoid-mediated HPA axis feedback resulting in greater HPA axis responsiveness [reviewed in Francis et al. (13)].

The differences in anxiety-like behavior as measured in the open-field test in peripubertal animals were somewhat different: although hypersensitive MS180 rats showed more evidence for anxiety (as indexed by the behavioral measures of crossovers and by thigmotactic behavior) than those without visceral hypersensitivity, it was the NH group that showed the greatest degree of anxiety-like behavior, whereas the other two groups did not differ from one another. These findings suggest that the central circuits associated with certain acute stress responses (e.g., SIVH and colonic motility) are not identical to those involved in a specific anxiety-like behavior. Another explanation may be that the differences between the three groups were assessed at the peripubertal age (6 wk), whereas the differences among MS180 rats with and without SIVH were assessed in adult animals. The peripuberal phase in rats is characterized by considerable neuroplasticity, and a direct correlation of behaviors exhibited during this phase with those in the adult phase is potentially problematic.

Is the SIVH in MS180 Rats Sensitive to Antagonism of the CRF/CRF1R System?

Our findings clearly indicate a role for enhanced CRF/CRF1R signaling in the mediation of acute and delayed SIVH in the MS180 animals. The fact that the nonselective CRF1 antagonist astressin B (46) was effective when given intracereurally in preventing acute and delayed SIVH suggests that a central CRF-R is involved in this inhibition. The selective CRF1R antagonist CP-154,526 (57), when given before the acute stressor, was able to prevent the development of delayed SIVH. Similar to what we have previously demonstrated in Wistar rats (49), this suggests an upregulation of the central CRF/CRF1R signaling system 24 h post-WA, followed by the activation of the system by the stress of the CRD procedure on day 2. Evidence for an acute stress-induced upregulation of the CRF/CRF1R system.
CRF$_2$R gene has previously been provided (28, 29, 45). Our findings suggest that this acute stress-induced upregulation of the CRF/CRF$_2$R system may be partly mediated by the neonatal rearing conditions.

Maternal separation of newborn rats is accompanied by a compromised ability to restrain the synthesis and the release of CRF in response to acute stressors (41, 42). Soderholm et al. (51) previously provided evidence that the acute stress-induced increase in colonic permeability was blocked by α-helical CRF$_9$–41, a nonselective CRF-R antagonist that does not cross the blood-brain barrier. It is therefore conceivable that both the central as well as the peripheral CRF/CRF$_2$R system can be upregulated as a consequence of aversive neonatal conditions,-mediated well-characterized central effects of the CRF system, such as stress-induced increase in colonic motility, increased anxiety-like behavior, and increased HPA axis responsiveness (22, 55) as well as peripheral effects related to colonic motility and permeability (57) and immune functions (52).

Although there is now evidence from several laboratories that the CRF system is involved in stress-mediated visceral pain modulation (15, 37, 48), the exact sites and mechanisms underlying this CRF system involvement are unclear (24). Several mechanisms have been proposed to mediate adult SIVH following neonatal maternal separation. On the basis of differential responsiveness of stress-induced somatic and visceral pain modulation to naloxone, we had previously suggested alterations in the engagement of endogenous pain-modulation mechanisms, such as a reduced activation of opioid systems (9). A rearing condition-dependent alteration of endogenous pain-modulatory systems could also explain the observed stress-induced visceral hypoalgesia in rats with neonatal handling, an intervention previously shown to decrease stress responsiveness.

Another potential mechanism underlying the SIVH observed in the MS180 rats is a stress-induced immune change in the colonic mucosa resulting in a chronic inflammatory response of the colonic mucosa that, in turn, could lead to persistent sensitization of visceral afferent pathways or to alteration in vagally induced pain modulation systems. Soderholm et al. (51), using a different maternal separation paradigm (separation of individual pups for 180 min daily from PND 4-21) and a different rat strain (male Sprague-Dawley rats), reported that 30-min WA stress of the adult rats resulted in increased colonic permeability for macromolecules (51). Similarly, Rosztoczy et al. (47), using a maternal separation paradigm in Wistar rats involving separation of individual pups from their moms, have provided evidence for increased basal levels of colonic permeability associated with bacterial translocation into mesenteric lymph nodes, liver, and spleen. These alterations were associated with an increase in colonic inflammation as indexed by an increased myeloperoxidase activity, mucosal mast cell density, and cytokine RNA expression (47). Because we did not evaluate colonic tissue from hypersensitive rats in the current study, we cannot rule out a role for colonic inflammation or mast cells in the development of visceral hyperalgesia in maternally separated animals. However, it must be kept in mind that these investigators used a significantly different maternal separation paradigm and different rat strains. Finally, it cannot be ruled out that the bulbospinal reflex underlying the VMR may be modulated centrally by stress-mediated mechanisms, thus producing an increased VMR that is not related to true alterations in visceral afferent pathways.

**Limitations of the Study**

The results of this study have to be interpreted with several potential limitations in mind. Tests of different aspects of the stress response (anxiety-like behavior, fecal pellet output, and VMR) were assessed at different ages and may, therefore, measure aspects of the stress system at different stages of development. In particular, anxiety-like behavior was assessed in the peripubertal age, a period of extensive neural development, significantly different from the situation in the adult animal. Along the same line, the stress experience of the young animals could have contributed to the adult phenotype. Sham WA experiments were not performed in the current study, but, as previously demonstrated both in MS180 rats (9) as well as in male Wistar rats (49), sham WA has no effect on VMR to CRD. Specific maternal behaviors, which have previously been demonstrated to be highly predictive of adult stress responsiveness (6) and which vary significantly between different moms (7), were not recorded. Although there was no difference in the number of hyper- and hyporesponsive MS180 rats between litters, we can only speculate that the differences between these groups may have been due in part to variations in the changes in maternal behavior induced by maternal separation. Because animals from nine litters subjected to the maternal separation paradigm were studied, a bias resulting from a particular dam’s maternal behavior is unlikely.

The variability in the expression of SIVH in the adult rat and the inability of a behavioral test performed at a younger age to predict the development of SIVH later in life limits the usefulness of this model as a simple animal model for IBS drug evaluation. On the other hand, this variability is reminiscent of the human condition, where IBS does not develop in every individual with a history of traumatic early life events but where the adult disease manifestation is influenced by a variety of other, partially known factors.

In summary, the current study confirms previous observations about the influence of rearing conditions on stress responsiveness (21) and on the development of SIVH in the adult rat (9). Our data indicate that the expression of an altered adult response to an acute stressor in form of SIVH is dependent on the activation and acute stress-induced upregulation of the central CRF/CRF$_2$R system. CRF$_2$R antagonists are currently in development for the treatment of IBS (55), and the current observations would predict that these agents may have a beneficial action both on acute stress effects as well as on the delayed manifestations of stressful life events.

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Present address of S. V. Coutinho: Janssen Pharmaceutica, 1621 Stony Circle, Quakertown, PA 18951.

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